Expression, Purification and Partial Characterization of Recombinant Gloshedobin, a Thrombin-like Enzyme from The Venom of *Gloydius shedaoensis* *

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Abstract The cDNA of gloshedobin was synthesized and amplified by RT-PCR from the total RNA of snake (*Gloydius shedaoensis*) venom gland. The 711 bp nucleotide sequence, which encodes the mature gloshedobin, was cloned into expression vector pPIC 9K and transferred into yeast *Pichia pastoris*, strain GS115. Transfermants with phenotype His^+ Mut^+ were selected to study their expression. Recombinant protein was conveniently separated and purified from the supernatant by two chromatographic steps: ion exchange chromatography on Q-Sepharose FF and affinity chromatography on Benzamidine-Sepharose 4BCL. Like intact gloshedobin, the recombinant enzyme exhibited strong esterase activity using tripeptide *p*-nitroanilide derivatives as substrate, but hydrolyzed N-*p*-tosyl-L-arginine methyl ester (TAME) very weakly. The recombinant protein displayed extreme instability at 37 ℃ in neutral buffer but higher stability at 0 ℃, and also, pH is not a key factor to affect its stability while the optimal pH for its enzymatic activity is pH 8.0.

Key words thrombin-like enzyme, cloning, expression, purification, characterization

Gloshedobin is a thrombin-like enzyme obtained from the venom of *Gloydius shedaoensis*. In contrast with thrombin, which converts fibrinogen to fibrin by removing fibrinopeptides A and B, a thrombin-like enzyme releases only fibrinopeptide A from fibrinogen[1]. As it does not activate factor XIII, the formed clots are not cross-linked and are more susceptible to plasmin degradation resulting in a rapid clearance from the microcirculation[2]. Consequently, the thrombin-like enzyme appears to be an appropriate alternative to heparin and may be preferable to it in hemorrhagic complications. Thrombin-like enzymes have been studied widely because of their therapeutic potential in myocardial infarction and thrombotic diseases[3~5].

Although gloshedobin can be purified from the snake venom, it is difficult to obtain large amounts of it either for studies or for large-scale production. So far, many reports focus on *E. coli* expression system to produce thrombin-like enzymes. However, highly expressed heterogeneous proteins in *E. coli* tend to form biologically inactive inclusion bodies combined with difficulties in refolding. Ineffective refolding will lead to very low activity recovery, sometimes as low as 3%[6,7]. Over the last few years, the methylotrophic yeast *Pichia pastoris* has been developed into a powerful expression system for a number of foreign genes[8]. Unlike *E. coli* system, this eukaryotic system has several advantages, such as: 1) ability for rapid growth to high cell density; 2) ability to express soluble heterogeneous proteins; 3) ability to modify expressed proteins. In this work, cDNA of gloshedobin was synthesized and amplified by RT-PCR from the total RNA of snake (*Gloydius shedaoensis*) venom gland. Then the gene of mature gloshedobin was cloned and expressed in the methylotrophic yeast *Pichia pastoris*. Finally, the recombinant protein was purified and partially characterized.

1 Materials and Method

1.1 Synthesis and amplification cDNA of gloshedobin by RT-PCR

Freshly prepared total RNA (1 μg) from the snake venom of *Gloydius shedaoensis*, was incubated at 65 ℃ for 10min and then transferred on ice immediately. Synthesis of cDNA was conducted according to manufacturer's protocol (Gibco, USA). Based on homology[5,9~11], the obtained cDNA was amplified by PCR using two primers respectively: 5′-ATGGTGCTGATCAGMGTG-3′ (M = A + T) and 5′-CGCCCAGGGTTTTCCCAGTCACG AC-3′. RT-PCR kit, 5′-full RACE Core Set (Takara, Japan), was used for RT-PCR to obtain the full length nucleotide sequence. The nucleotide sequences were determined by the dideoxy chain-termination method.

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1.2 Construction of expression vector

The mature gloshedobin gene was amplified by PCR to place *Eco*R I site at its 3′ terminal and place α-factor signal sequence, ending at cleavage site *Ste*13, at its 5′ terminal. The two applied primers are: 5′-GAAAAGAGAGGCTGAAGCTATCATTGGAGGTGATGAATG-3′ and 5′-CCGAATTCTTATCATGGGGGGCAGGTTGCA-3′, respectively.

1.3 Expression recombinant protein in shake flask

The construct was integrated into the genome of yeast *Pichia pastoris* strain GS115 (Invitrogen, USA) by electroporation. Transfermants with phenotype His^+ Mut^+ were selected. Culture condition and expression induction were manipulated according to manufacturer's instructions(Invitrogen, USA). Expression of gloshedobin was determined by examining enzymatic activity.

1.4 Purification procedure

The pH of the culture was adjusted to 5.0 and then centrifuged at 17 000 *g* (Ricoh, Japan) for 30 min to remove cells and precipitates. The supernatant was fractioned by ion exchanger on Q Sepharose FF (Pharmacia, Sweden) and the active peak was eluted at the gradient of 0~0.5 mol/L NaCl in 20 mmol/L methylpiperazin-HCl, pH 5.0 in 20 column volume. Then the obtained active material was purified by affinity chromatographic adsorbents on benzamidine-Sepharose 4BCL (Pharmacia, Sweden) in 50 mmol/L Tris-HCl, pH 8.2, eluting with 0.5 mol/L NaCl in the same buffer. The active products eluted from ion exchanger were loaded to affinity chromatographic matrix on ConA Hitrap (Pharmacia, Sweden) to examine post-modification, specifically eluted by 0.5 mol/L methyl-α-D-glycopyanoside in 20 mmol/L Tris-HCl, pH 7.4, containing 0.5 mol/L NaCl.

1.5 Amidolytic activity assay

Amidolytic activity was assayed using chromogenic substrate (Nα-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide, Sigma)[12]. One unit of amidolytic activity was defined as the amount of enzyme necessary to hydrolyze 1.0 μmol of substrate per min. Ancrod, a thrombin-like enzyme from *Calloselasma rhodostoma* (Sigma), was used as standard enzyme.

1.6 Arginine esterase assay

Arginine esterase activity was measured by the method described by Yabuki *et al.*[13], using N-*p*-tosyl-L-arginine methyl ester (TAME) (Sigma) as substrate.

1.7 Protein concentration

Protein concentration was determined by the method of BCA[14].

1.8 SDS-PAGE electrophoresis

All the electrophoresis were manipulated according to the manual using PhastGel System (Pharmacia, Sweden).

1.9 N-amino acid sequence analysis

The first 10 N-terminal residues of recombinant protein were determined by automatic Edman degradation procedure.

2 Results and Discussion

2.1 Construction of expression vector

The expression vector pPIC 9K was constructed to encode mature gloshedobin, which contains 237 amino acids, 711 base pairs (Figure1a). The fragment of gloshedobin gene was cloned in frame within the secretion signal open reading frame and inserted within signal cleavage site *Ste*13 (Figure1b).

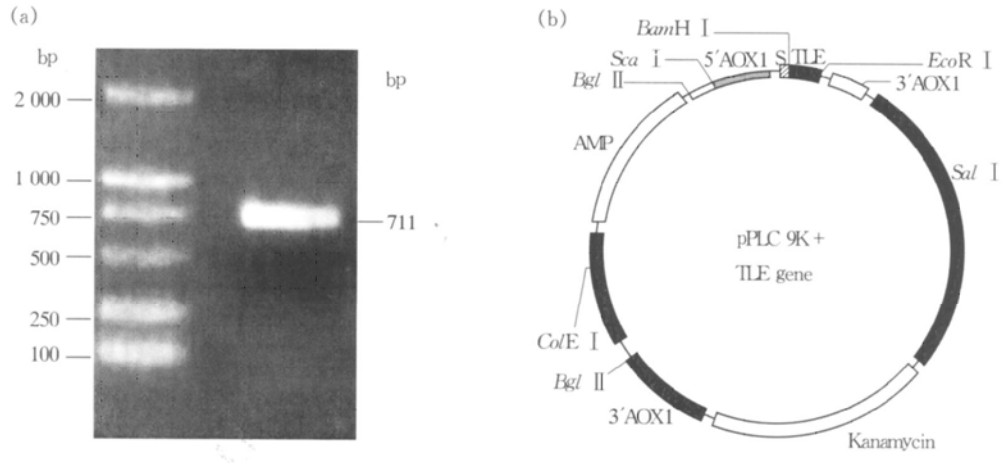

Fig. 1 Expression construct of gloshedobin gene

(a) agarose gel electrophoresis analysis of gloshedobin gene; (b) schematic diagram of expression vector construction.

2.2 Expression and purification of recombinant gloshedobin

Transfermants with phenotype His^+ Mut^+ were applied to study the expression of gloshedobin in 500 ml shaking flask. Having been induced with methanol for 36 hours, the target enzyme could be

examined in the supernatant by measuring amidolytic activity. Compared with minimal medium, rich medium led to higher expression level of 10 mg target protein per liter culture.

The recombinant gloshedobin was conveniently purified by three steps (Figure2). Firstly, the culture was adjusted pH to 5.0 in order to remove cells and precipitates. This is because the recombinant gloshedobin's p*I* is around 3.5 ~ 4.5 according to isoelectric focusing electrophoresis (data not shown). Secondly, the prepared sample was separated and purified by a combination of chromatography: ion exchange chromatography on Q Sepharose FF and affinity chromatography on benzamidine Sepharose 4BCL. Benzamidine is a competitive inhibitor for serine proteases. As thrombin-like enzymes belong to serine protease family, the matrix: benzamidine-Sepharose 4BCL would probably specifically capture gloshedobin. Table 1 shows the recovery and efficiency of each separation step.

Table 1 Amidolytic and TAME activity of the active fractions obtained after the purification steps

Fractions	Protein		Amidolytic Activity				TAME
	Total /mg	Recovery /%	Total /U	Recovery /%	Specific activity /U·mg^{-1}	Purification fold(s)	activity /U·mg^{-1}
Culture	1 248	100	4 920	100	3.9	1	0.2
Q-Sepharose	300	24	3 936	81	13.1	3.4	0.66
Benzamidine	112	9	3 493	71	31.2	8.0	1.56

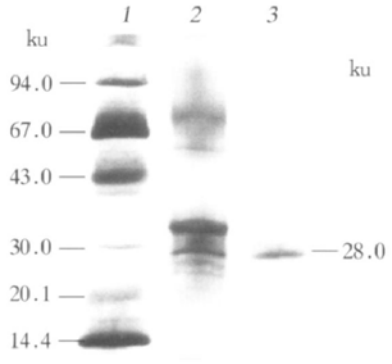

Fig. 2 Electrophoresis analysis of each chromatographic step
1: marker; *2*: active peak from Q-Sepharose FF; *3*: active products from benzamidine-Sepharose 4BCL.

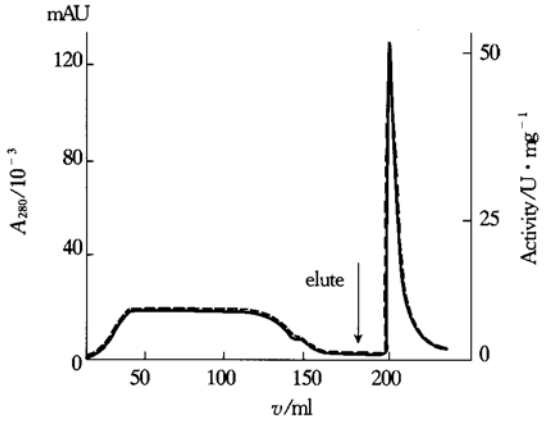

Fig. 3 Recombinant gloshedobin adsorbed to affinity column: ConA Hitrap
– – –: activity; ——: A_{280}. Chromatographic procedure, see section "material and method".

Intact gloshedobin from snake venom has a possible glycosylation site at Asn^{124}-Ser^{125}-Thr^{126} with respect to its amino acid sequence homology compared with other thrombin-like enzymes[5]. That their positively binding to affinity matrix, ConA Sepharose, indicates that glycosylation does happen to recombinant gloshedobin (Figure3). The reason is that there is an affinity interaction between lectin, the specific ligand of the matrix: ConA- Sepharose, and glycoprotein. However, the glycosylation occurring on which site and in which form is not clear yet.

2.3 Partial characterization of recombinant gloshedobin

2.3.1 N-terminal amino acid sequence analysis: The N-terminal amino acid sequencing of recombinant gloshedobin revealed a single sequence, Glu-Ala-Ile-Ile-Gly-Gly-Asp-Glu-Cys-Asn. Residues Ile-Ile-Gly-Gly-Asp-Glu-Cys-Asn correspond to N-terminal sequence of the native enzyme. And the two extra residues Glu-Ala were expected to be cleaved by restriction enzyme *Ste*13 automatically when travelling through the cell membrane. One possible explanation is that environmental components, for example, the advanced configuration of the heterogeneous protein, will probably interfere with enzyme *Ste*13. Furthermore, glycosylation at N-terminal may disrupt the secondary structure and shield the *Ste*13 cleavage site resulting in an intact form.

2.3.2 Stability: The recombinant enzyme in solution shows slightly difference of stability in the range of pH4.5 ~ 8. The optimal pH for enzymatic reaction is pH 8.0. However, compared with natural gloshedobin, this enzyme is much more sensitive to the temperature. Incubated overnight at 37 ℃ in 20 mmol/L Tris-HCl, pH 7.0 or 20 mmol/L methylpiperazin-HCl, pH 5.0, it will be autocleaved into peptides. Repeated freezing and thawing will greatly affect the activity of gloshedobin. According to the prediction of a computer program in Swiss-Model, its estimated half-life is: 30 min (yeast, *in vivo*). In this case, it is classified as unstable one.

2.3.3 Substrate specificity: Like natural gloshedobin, this recombinant enzyme exhibited strong esterase activity using tripeptide *p*-nitroanilide derivatives as substrate, but hydrolysed N-*p*-tosyl-L-arginine methyl ester (TAME) very weakly, roughly one twentieth of activity of hydrolysing the former substrate. Furthermore, the ability of the enzyme to hydrolysis two tripeptide *p*-nitroanilide derivatives, N-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide and N-benzoyl-Pro-Phe-Arg *p*-nitroanilide were studied. The enzyme showed as much as two times more active when using N-benzoyl-Pro-Phe-Arg *p*-nitroanilide as substrate than using the other one.

2.3.4 Molecular mass: The molecular mass of this enzyme, 27 675 u, is determined by mass spectrum (data not shown). According to SDS-PAGE, the molecular mass of this enzyme is about 28 ku. Compared with the theoretic molecular mass calculated by adding all amino acids, 26 000, the difference is probably contributed by the sugar chain. In this case, the percentage of the sugar moiety is about 7%.

References

1 Pirkel H, Theodor I. Thrombin-like venom enzymes: structure and function. Adv Exp Med Biol, 1990, **281**: 165~175

2 Matsui T, Fujimura Y, Titani K, *et al*. Snake venom proteases affecting hemostasis and thrombosis. Biochim Biophys Acta, 2000, **1477** (1~2): 146~156

3 Pfeiffer G, Dabrowski U, *et al*. Carbohydrate structure of a thrombin-like serine protease from *Agkistrodin rhodostoma*. Eur J Biochem, 1992, **205** (3): 961~978

4 Hahn B S, Yang K Y, Park E M, *et al*. Purification and molecular cloning of calobin, a thrombin-like enzyme from *Agkistrodon caliginosus* (Korean viper). J Biochem, 1996, **119** (5): 835~843

5 AU L C, Lin S B, Chou J S, *et al*. Molecular cloning and sequence analysis of the cDNA for ancrod, a thrombin-like enzyme from the venom of *Calloselasma rhodostoma*. Biochem J, 1993, **294** (Pt 2): 387~390

6 Maeda M, Satoh S, Suzuki S, *et al*. Expression of cDNA for batroxobin, a thrombin-like snake venom enzyme. J Biochem, 1991, **109** (4): 632~637

7 Selistre-de-Araujo H S, Souza E L D, Beltramini L M, *et al*. Expression, refolding and activity of a recombinant nonhemorrhagic snake venom metalloprotease. Protein Expr Purif, 2000, **19** (1): 41~47

8 Cregg J M, Vedvick T S, Raschke W C, *et al*. Recent advances in the expression of foreign genes in *Pichia pastoris*. Biotechnology (NY), 1993, **11** (8): 905~910

9 Pan H, Zhou Y C, Yang G Z, *et al*. Clonging and expression of cDNA for thrombin-like enzyme from *Agkistrodon halys* Pallas Snake venom. Acta Biochim Biophys Sin, 1999, **31** (1): 79~82
潘 华, 周元聪, 杨冠珍. 生物化学与生物物理学报, 1999, **31** (1): 79~82

10 Itoh N, Tanaka N, Mihashi S, *et al*. Molecular cloning and sequence analysis of cDNA for batroxobin, a thrombin-like snake venom enzyme. J Biol Chem, 1987, **262** (7): 3132~3153

11 Deshimaru M, Ogawa T, Nakashima K I, *et al*. Accelerated evolution of crotalinae snake venom gland serine proteases. FEBS Lett, 1996, **397**: 83~88

12 Lottenberg R, Christensen U, Hackson C M, *et al*. Assay for coagulation protease using peptide chromogenic and fluorogenic substrates. Methods in Enzymology, 1981, **80** (Pt C): 341~361

13 Yabuki Y, Oguchi Y, Takahashi H, *et al*. Purification of kininogenase-2 from the venom of *Agkistrodon caliginosus* (Kankokumamushi). Toxicon, 1991, **29** (1): 73~84

14 Smith P K, Krohn R I, Hermanson G T, *et al*. Measurement of protein using bicinchoninic acid. Analytical Biochemistry, 1985, **150** (1): 76~85

大连蛇岛蝮蛇类凝血酶基因在毕赤酵母中的克隆表达及分离纯化*

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摘要 根据同源性分析设计引物, 通过 RT-PCR 方法从大连蛇岛蝮蛇毒腺总 RNA 中合成扩增出类凝血酶基因, 之后将该基因克隆到表达载体 pPIC9K 中, 经电激转化后整合至毕赤酵母细胞基因组中. 经筛选得到甲醇快速生长型转化子 His^+ Mut^+ 在 500 ml 摇瓶中培养, 甲醇诱导分泌表达. 上清液中重组类凝血酶是通过两步柱层析得到: Q Sepharose FF 和 Benzamidine-Sepharose 4BCL. 与天然蛇毒类凝血酶一致, 分泌表达的重组类凝血酶具有较强的酯酶活性, 但精氨酸甲酯如 TAME 的水解活性较弱. 此重组类凝血酶在 37 ℃中性溶液中保存过夜将分解成小肽, 但在 0 ℃下很稳定. 该酶的最适 pH 为 8.0.

关键词 类凝血酶, 克隆, 表达, 纯化, 特性表征

学科分类号 Q786

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